Year: 2014

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DOI: https://doi.org/10.1016/j.jim.2014.04.009

Originally published at:
Chatterjee, Bithi; Leung, Carol Sze; Münz, Christian (2014). Animal models of Epstein Barr virus infection. Journal of Immunological Methods, 410:80-87.
DOI: https://doi.org/10.1016/j.jim.2014.04.009

Animal models of Epstein Barr virus infection

Bithi Chatterjee, Carol Sze Leung and Christian Münz*

Viral Immunobiology, Institute of Experimental Immunology, University of Zürich, Switzerland

*Corresponding author: Christian Münz, Viral Immunobiology, Institute of Experimental Immunology, University of Zürich, Winterthurerstrasse 190, 8057 Zürich, Switzerland, e-mail: christian.muenz@uzh.ch, Tel.: +41-44-635-3716, Fax: +41-44-635-6883

Keywords: infectious mononucleosis; dendritic cells; T cells; NK cells; lymphoproliferative disease; humanized mice; EBV-associated lymphomas

Highlights:

- EBV establishes persistent infection in HIS mice.
- Pathological EBV latency III lymphomas and lytic replication develop in HIS mice.
- HIS mice mount innate and adaptive cellular immune responses to EBV.

Abstract

Epstein Barr virus (EBV) was the first human tumor virus to be identified. Despite 50 years of research on this oncogenic virus, no therapeutic or prophylactic vaccine is available against this pathogen. In part, the development of such a vaccine is hampered by the lack of in vivo models for EBV infection and immune control. However, with the advent of mice with reconstituted human immune system components (HIS mice), certain aspects of EBV associated diseases and immune responses can be modeled in vivo. In this review, we will discuss the insights that can be gained from these experiments, and how immune system components can be manipulated to interrogate their function during EBV infection. Finally, we will compare EBV immunobiology in HIS mice to infection by EBV-related viruses in monkeys, and we will outline the strengths and weaknesses of these two in vivo models of EBV infection. Both of these models show great promise as a platform for preclinical EBV vaccine testing.

1. Introduction

Epstein Barr virus (EBV) is a common gamma herpesvirus in the $\gamma 1$ subgroup, which is composed of lymphocryptoviridae, and it was identified as the first human candidate tumor virus in 1964 (Epstein et al., 1964). Since then, it has been associated with many important human malignancies of lymphocyte and epithelial cell origin, such as Burkitt's lymphoma, Hodgkin's B cell lymphoma, and nasopharyngeal carcinoma (Kutok and Wang, 2006). Therefore, the interest in EBV's biology, particularly tumorigenesis and immune control, is considerable and *in vivo* models for EBV infection could provide significant insights into these topics.

However, this issue is complicated by the apparent lack of lymphocryptoviruses in rodents (Ehlers et al., 2008; Ehlers et al., 2010). The $\gamma 1$ herpesvirus subfamily has only developed in monkeys, and these viruses have evolved closely with their hosts, namely new and old world monkeys as well as primates. The closest relative of EBV in mice is the $\gamma 2$ rhadinovirus murine γ-herpesvirus 68 (MHV-68). Unfortunately, it lacks the acute growth transforming ability of EBV and therefore cannot recapitulate EBV induced tumorigenesis (Virgin and Speck, 1999; Flano et al., 2002; Barton et al., 2011). In addition, MHV-68 persists in myeloid cells as well as in epithelial cells and B cells, which are the main host cells of EBV in the human body. Indeed, viral latency seems to be established in a significant proportion of macrophages and dendritic cells. Moreover, symptomatic primary MHV-68 infection expands a characteristic $CD8^+$ T cell subset with $V\beta 4$ positive T cell receptors, which is not MHC restricted, but requires latently infected B cells for its expansion (Flano et al., 2002). Thus, apart from the molecular differences between these distantly related gamma herpesviruses, major differences in tropism, tumorigenesis, and immune responses exist between EBV and MHV-68 infection. Therefore, alternative in vivo models for EBV infection are required to understand the basic biology of this important human pathogen, which is estimated to cause around 2% of all human tumors (Cohen et al., 2011). Moreover, a model that would faithfully reproduce EBV-specific immune control could be used to develop and evaluate vaccine candidates against this human oncogenic virus.

2. Mice with reconstituted human immune system components (HIS mice)

One such model would be mice with reconstituted human immune system components (HIS mice). These are generated using mouse strains with genetic lesions that compromise the development of mouse lymphocytes (Leung et al., 2013a; Rongvaux et al., 2013). Most commonly the non-obese diabetic (NOD) or the BALB/c mouse strain with *scid* or RAG mutations are used. These animals cannot somatically rearrange or repair B and T cell receptors and thus, lack adaptive lymphocytes. In addition, deletion of the common gamma chain (γ_c) of interleukin (IL)-2, -4, -7, -9, -15, and -21 signaling abolishes innate lymphocyte development, and primarily compromises IL-15 dependent natural killer (NK) cell maturation. The earliest models transferred mature human immune system components such as peripheral blood mononuclear cells (PBMCs) into these mice (Legrand et al., 2006). However, this transfer leads to graft-versus-host-disease (GvHD) by the human immune cells, because they were not tolerized against their new mouse host. The usefulness of such PBMC transfer models is limited, because reconstitution by transferring large human cell numbers accelerates demise by GvHD. Alternatively, human CD34 positive hematopoietic progenitor cells (HPCs) can be transferred into these mouse backgrounds. Most frequently, BALB/c RAG2$^{-/-}$ $\gamma_c^{-/-}$ (BRG) or NOD-*scid* $\gamma_c^{-/-}$ (NSG) mice are used for these experiments (Traggiai et al., 2004; Ishikawa et al., 2005; Shultz et al., 2005) (Figure 1). These animals reconstitute most human immune system compartments several months after HPC introduction, but lack significant gut mucosa colonization and germinal center development. Gut colonization can be improved by similarly reconstituting NOD-*scid* mice together with the additional transfer of a human fetal thymus and liver organoid under the kidney capsule (BLT mice) (Nochi et al., 2013). This organoid seems to educate the reconstituted human T cell repertoire to develop certain specificities that are more similar to humans (Jaiswal et al., 2012), but compromises tolerance induction against the mouse host at the same time (Covassin et al., 2013). Therefore, NSG and BRG mice with reconstituted human immune system components as well as BLT mice (huNSG or huBRG, collectively HIS mice) are currently used to investigate human pathogens with a tropism for the hematopoietic lineage. These models are also currently being explored for studies of EBV infection and immune control in vivo.

3. Programs of EBV infection in HIS mice

The exclusive tropism of EBV for human cells is a hurdle that has been overcome with the usage of HIS mice. EBV robustly infects HIS mice, compared to non-reconstituted animals (Traggiai et al., 2004). EBV infection in humans has lytic and latent infection stages. Lytic infection produces infectious virus for transmission to other susceptible host cells within the infected individual, mainly epithelial and B cells, or to uninfected individuals during transmission via saliva exchange (Young and Rickinson, 2004). EBV infection is mediated by multiple virally encoded glycoproteins that act on specific cell types. B cell infection is facilitated by the interaction of gp350 (BLLF1) with the complement receptors CD21 or CD35, and gp42 (BZLF2), binding to MHC class II molecules, together with integrin targeting gH (gp85) and gL (gp25) promote viral fusion. Epithelial cell entry is less well characterized, but involves the viral protein BMRF2, which binds epithelial cells, as well as the gL (gp25)/gH (gp85) fusion complex. Epithelial cell infection is thought to be mainly mediated by integrins (Shannon-Lowe and Rowe, 2014). While epithelial cells are thought to primarily sustain lytic replication, B cells can harbor all latent and lytic infection programs (Thorley-Lawson and Gross, 2004). In B cells, EBV expresses different latent and lytic gene products depending on the differentiation stage of the host cell. While infected naïve human B cells primarily express all eight latent EBV proteins (six nuclear antigens or EBNAs and two latent membrane proteins or LMPs; called latency III), infected germinal center B cells express EBNA1, LMP1, and LMP2 (latency II), infected homeostatically proliferating memory B cells express only EBNA1 (latency I), and non-proliferating infected peripheral memory B cells express no EBV proteins (latency 0) (Babcock et al., 1998; Babcock et al., 2000; Hochberg et al., 2004). Infected plasma B cell differentiation reactivates EBV into lytic replication

(Laichalk and Thorley-Lawson, 2005). Since HIS mice lack human epithelial cells, mainly B cell infection by EBV can be assessed in this in vivo model.

Both latent and lytic EBV antigen expression can be observed in infected HIS mice (Strowig et al., 2009; Chijioke et al., 2013; Tsai et al., 2013). Depending on the dose of EBV, it is possible to generate asymptomatic persistent infection or acute infectious mononucleosis-like symptoms, as well as lymphoproliferative disease in HIS mice (Yajima et al., 2008). Latency III expressing cells can be found in all of these instances, as assessed by immunohistochemistry and in situ hybridization for EBV-encoded RNAs (EBERs), EBNA1, EBNA2, and LMP1 (Cocco et al., 2008; Strowig et al., 2009). However, it has been suggested that asymptomatic persistent infection without the massive lymphocyte expansion of infectious mononucleosis can also result in lower latency stages (Islas-Ohlmayer et al., 2004; Cocco et al., 2008; Ma et al., 2011). This assessment was primarily done by immunohistochemistry and requires confirmation by other methods such as EBV transcript and promoter usage analysis. Along these lines, a recent study described T cell dependent Q promotor usage of EBNA1 in HIS mice (Heuts et al., 2014). EBNA1 transcription from the Q promotor only occurs during latencies I and II. This could indicate that a low level of T cell interaction with B cells in HIS mice allows for the access of lower EBV latency programs. Nevertheless, persistent infection with EBV in HIS mice does not depend on lytic replication, because EBV lacking BZLF1, the immediate early transactivator of lytic replication, can still establish infection in HIS mice (Ma et al., 2011; Chijioke et al., 2013). With respect to lytic replication, latent EBV-infected B cells far outnumber lytic EBV antigen expressing cells in HIS mice that have been infected with the B95-8 EBV strain, a clinical isolate from an American infectious mononucleosis patient (Miller and Lipman, 1973; Strowig et al., 2009; Chijioke et al., 2013). In these rare, lytically EBV replicating cells however, both early and late lytic antigen expression can be found (Chijioke et al., 2013). Accordingly, both cell-associated and plasma viral DNA loads can be detected in EBV infected HIS mice (Chijioke et al., 2013). However, it remains unclear how much of the plasma viral DNA load is infectious or whether it could merely be DNA released from dying infected cells. In addition to this prototypic laboratory EBV strain, the EBV strain M81, derived from a nasopharyngeal carcinoma patient and which displays an enhanced tropism for epithelial cells and a reduced tropism for B cells, was investigated in HIS mice. All mice infected with the same infectious dose of M81 showed elevated EBV lytic replication in B cells compared to the wild type B95-8, in line with the enhanced viral replication observed in nasopharyngeal carcinoma patients (Tsai et al., 2013). M81 infected HIS mice expressed gp350, a viral glycoprotein that EBV uses for attachment to B cells during entry (Tsai et al., 2013). Therefore, it is clear that EBV establishes latency III and lytic replication in HIS mice after infection. The assessment of additional latency programs in this vivo model awaits further investigation and confirmation.

4. EBV-associated diseases in HIS mice

EBV is associated with a number of human malignancies including Burkitt's lymphoma, Hodgkin's lymphoma, nasopharyngeal carcinoma, and post-transplant lymphoproliferative disease, which have been characterized with respect to EBV protein expression and latency type. The development of certain EBV-associated diseases can be studied in HIS mice. Infection with a high dose of EBV wild-type strain B95-8 leads to lymphoproliferative disease and tumor generation (Yajima et al., 2008; Strowig et al., 2009). B cells primarily harbor the virus and can be purified from EBV-infected HIS mice and cultured in vitro as immortalized lymphoblastoid cell lines (LCLs). They express all eight latent EBV antigens and are classified as latency III, including EBV latent nuclear antigen 3B (EBNA3B). Infection by EBNA3B-deficient EBV mutant virus leads to more aggressive proliferation of B cells and causes diffuse large B cell lymphomas in HIS mice (White et al., 2012). The gene expression profile of in vivo transformed tumor cells is similar to that of immortalized lymphoma lines from a subset of diffuse large B cell lymphoma patients that have EBNA3B-mutated EBV in their tumor cells. The authors also found that T cells in EBNA3B-deficient EBV infected mice failed to infiltrate tumor tissues. This might be due to a more rapid expansion of EBNA3B-deficient B cells and poorer secretion of the T cell attracting chemokine CXCL10, reducing T cell recruitment in vitro and T cell mediated killing in vivo. The genetic mechanism by which this occurs is under investigation. A recent study demonstrated that the EBNA3A protein could mediate silencing of the CXCL10 locus in vitro (Harth-Hertle et al., 2013). This indicates that different EBNA3 members regulate this important chemokine diametrically. EBV deficient in the lytic protein BZLF1 was also studied in HIS mice. One study showed that BZLF1-deficient EBV infection caused less EBV-associated lymphomas (Ma et al., 2011). These studies suggest that HIS mice recapitulate lymphomagenesis upon EBV infection and allow for the examination of different viral isolates and mutants for their tumor formation capacity.

Besides lymphomas, it has been reported that EBV infection in HIS mice leads to hemophagocytic lymphohistiocytosis and erosive arthritis (Kuwana et al., 2011; Sato et al., 2011). Both studies used the EBV strain Akata to infect NOD/ShiJic-*scid* $\gamma_c^{-/-}$ (NOG) mice reconstituted with $CD34^+$ human HPCs. Sato et al. demonstrated that two-thirds of the infected mice died 10 weeks post-infection after exhibiting high and persistent viremia, leukocytosis, IFNγ cytokinemia, normocytic anemia, and thrombocytopenia, which imitate the immunopathologic aspects of EBV-associated hemophagocytic lymphohistiocytosis in humans (Sato et al., 2011). In patients, EBV has been implicated in the pathogenesis of autoimmune diseases, including multiple sclerosis, systemic lupus erythematosus, and rheumatoid arthritis (Munz et al., 2009). Kuwana et al. reported the development of erosive arthritis accompanied by synovial membrane proliferation, pannus formation, and bone marrow edema in 15 of 23 mice infected with EBV. However, this finding was only based on the histology of joint and adjacent bone marrow tissues in infected mice; markers of rheumatoid arthritis were absent in these mice (Kuwana et al., 2011). These studies suggest that other EBV-associated diseases causing hyperinflammation and autoimmunity might be modeled in HIS mice. However, lymphoma formation is still the most accessible disease manifestation after EBV infection in this in vivo model.

5. Innate immune responses to EBV in HIS mice

For some of these EBV-associated diseases, the course of the primary infection with this virus is decisive. While EBV establishes persistent infection usually without symptoms in small children, adolescents suffer frequently from infectious mononucleosis (IM) after primary EBV infection (Luzuriaga and Sullivan, 2010). IM confers increased risks for developing EBV associated Hodgkin's lymphoma and multiple sclerosis (Hjalgrim et al., 2003; Thacker et al., 2006; Nielsen et al., 2009). Thus, it is important to understand why EBV causes IM when primary infection is delayed. IM is accompanied by high viral loads, including elevated shedding of viral particles into the oropharynx. This increased lytic replication is also reflected by the massive expansion of lytic EBV antigen-specific $CD8^{+}$ T cells that cause enlarged secondary lymphoid tissues like tonsils and spleen, and which probably induce IM symptoms by their elevated cytokine production. Lack of sufficient innate immune control of EBV in patients could be a cause for the initial uncontrolled lytic replication, which can only be brought under control with an immunopathological $CD8^{+}$ T cell response. These immune responses can be studied further using HIS mice. Among the innate immune compartments, dendritic cells (DCs) and NK cells are well reconstituted in huNSG mice and the low frequencies of NK cells in huBRG mice can be expanded by IL-15 injections (Huntington et al., 2009; Strowig et al., 2010; Meixlsperger et al., 2013). Type I IFN secreting plasmacytoid DCs have been shown to restrict EBV infection in a PBMC transfer model (Lim et al., 2006), although IFN-α/β might inhibit EBV infection only very early after viral encounter with B cells (Lotz et al., 1985). The role of other DC populations, which are required to prime protective EBV-specific T cells in vitro (Bickham et al., 2003), has not been investigated in an in vivo model of EBV infection so far. Another group of innate leukocytes, namely NK cells, are thought to restrict EBV infection initially. Genetic lesions causing susceptibility to EBV infection in primary immunodeficiency patients have pointed to compromised NK cell responses (Biron et al., 1989). However, all so far identified mutations affect other immune compartments

(Mace et al., 2013), and NK cell depletion did not alter MHV-68 infection (Usherwood et al., 2005). In order to determine the role of NK cells during primary EBV infection, NK cell depletion prior to EBV infection was recently analyzed in huNSG mice (Chijioke et al., 2013). HuNSG mice lacking NK cells had higher EBV titers and presented signs of elevated lytic replication (higher serum viral load and lytic antigen expression in spleen sections). Only infection with wild type, but not lytic replication deficient virus was affected by NK cell depletion and drove early differentiated NK cell expansion in huNSG mice. The elevated lytic replication of wild-type virus in the absence of NK cells led to increased $CD8^{+}$ T cell expansion and splenomegaly. Furthermore, elevated monoclonal lymphoproliferation and weight loss were observed as clinical symptoms in EBV-infected and NK cell depleted huNSG mice. These data suggest that the early differentiation stage of NK cells in huNSG mice restricts lytic EBV replication. In the absence of this innate immune control, EBV infection causes higher viral titers, driving massive $CD8^{+}$ T cell expansion and causing IM symptoms. Therefore, it is tempting to speculate that the incompletely differentiated NK cell repertoire in children's' immune systems copes with lytic EBV replication better and prevents IM early in life.

6. Adaptive immune responses to EBV in HIS mice

Adaptive immune responses to EBV are being investigated in HIS mice in vivo. B cell responses have been investigated in these mice with varying degrees of success. In HIS mice, immunoglobulin responses are generally weak due to poor germinal center organization and inefficient class switching. IgM and IgG have been observed at low levels several months after reconstitution (Traggiai et al., 2004; Ishikawa et al., 2005; Shultz et al., 2005), but EBV-specific responses have been observed infrequently; only EBV-specific IgM (Yajima et al., 2008) was observed after infection. Efforts are underway to understand and enhance B cell responses in these animals, including the use of inflammatory DCs to improve lymph node formation or enzymes to facilitate immunoglobulin class switching. Additionally, the generation of MHC Class

II transgenic animals may facilitate T cell/B cell cross talk. Such improved interactions could both strengthen humoral immune responses in HIS mice and promote EBV latency II through germinal center B cell differentiation.

In contrast, in response to EBV infection, HIS mice develop $CD4^+$ and $CD8^+$ T cells (Traggiai et al., 2004; Strowig et al., 2009) with lytic and latent antigen specificities that are similar to humans, including recognition of the T cell epitopes $BRLF1_{109-117}$, $BMFL1_{280-288}$, and $LMP1_{159-167}$, in animals containing HLA-A*0201 transgenes (Strowig et al., 2009; Shultz et al., 2010). Animals without HLA transgenes also develop EBV specific T cells, but these are often specific for subdominant epitopes as characterized in humans. Animal with HLA transgenes allow for the thymic selection of T cells on these molecules and result in similar epitope specificities as in patients. Regardless of the source, these T cells produce IFN-γ in response to cognate $CD8^+$ T cell epitopes (Strowig et al., 2009) and autologous LCLs (Traggiai et al., 2004; Melkus et al., 2006; Yajima et al., 2008). Moreover, both $CD4^+$ and $CD8^+$ T cell clones that were primed during EBV infection of HIS mice are able to lyse LCLs (Strowig et al., 2009). Blocking recognition via MHC I or MHC I/II specific antibodies ex vivo results in reduced immune responses (Melkus et al., 2006; Yajima et al., 2008; Strowig et al., 2009). T cells are also important for the control of EBV and EBV-associated tumors in HIS mice and for their subsequent survival (Strowig et al., 2009; Yajima et al., 2009); depleting $CD8^+$ T cell populations increases tumor burden and increases viral loads in HIS mice, indicating that functional $CD8^+$ T cells are required for tumor control in vivo (Strowig et al., 2009). Interestingly, $CD4^+$ depletion alone also resulted in increased viral loads with minimal changes in tumor burden; depleting both T cell subsets resulted in the highest viral loads. These data indicate that both $CD4^+$ and $CD8^+$ T cell responses are involved in the immune control of EBV and EBV-associated tumors in vivo; $CD4^+$ T cells may be required for $CD8^+$ T cell help, and $CD8^+$ T cells are in turn needed for additional viral control. Indeed, adoptively transferring $CD8^+$ T cells to patients with lymphoproliferative disease ameliorated disease symptoms (Gottschalk et al., 2005). Many questions remain that can be answered using the HIS mouse model. It will be of interest to examine anti-EBV T cell activation in this disease model. For example, in HIS mice reconstituted with EBV^{+} adult donor PBLs, administering anti-CTLA4 antibodies resulted in prolonged survival by delaying lymphoproliferative disease (May et al., 2005), presumably by activating T cell immunity. The development of HIS mice with multiple HLA transgenes will allow for the additional exploration of T cell repertoires and their impact on EBV pathogenesis. Future studies to determine the functionality and role of different T cell populations in the pathogenesis of EBV and EBV-associated tumors will be of great interest for the treatment of EBV-associated diseases.

7. HIS mice as a platform to assess EBV specific vaccine formulations

HIS mice not only serve as preclinical surrogate models to investigate EBV-associated diseases and their immune control, but also act as a platform to examine vaccine formulations against EBV infection or EBV-related diseases. A vaccine formulation using a monoclonal antibody to target the human endocytic receptor DEC-205 for delivery of the EBV nuclear antigen 1 (EBNA1) was first tested in HIS mice by our group (Gurer et al., 2008). EBNA1 is the only latent protein of EBV that is expressed in all EBV-associated malignancies. This vaccination strategy efficiently delivers antigens to DCs and B cells and leads to efficient antigen processing and presentation (Leung et al., 2013b). Vaccination with an EBNA1 fusion monoclonal antibody specific for DEC-205 (αDEC-205-EBNA1) together with the Toll-like receptor 3 (TLR3) ligand polyIC as an adjuvant primed EBNA1-specific IFNγ secreting T cells and induced anti-EBNA1 IgM responses in a subset of immunized mice. In order to characterize EBNA1-specific T cell responses, we cloned the EBNA1-specific T cells from the splenocytes of responding immunized HIS mice. All of the obtained clones were $CD4^{+}$ T cells with a broad range of epitope specificities. Several of these EBNA1-specific $CD4^{+}$ T cell clones were able to recognize autologous LCLs as demonstrated by IFNγ secretion and CD107a degranulation (Meixlsperger et al., 2013). In the same study, different TLR ligand adjuvant formulations were evaluated in HIS mice. CD141$^+$ DCs produce IL-12 and large amounts of IFNα after TLR3 stimulation with polyICLC, a more stable form of polyIC. This particular DC subset corresponds to the mouse CD8$^+$ DCs known for their superior antigen cross presentation ability after maturation, and they can be similarly targeted by αDEC-205. The next step in these studies will be to test whether this vaccine formulation can confer protection against EBV infection in HIS mice, and if heterologous boost regimens allow the priming of EBV specific CD8$^+$ T cells. Nevertheless, studies thus far suggest that antigen formulations and adjuvants can be tested for their stimulatory capacity in vivo in HIS mice.

8. Lymphocryptoviruses in monkeys

Even though HIS mice offer the possibility to interrogate certain aspects of EBV infection and immune control in vivo, they have also significant limitations in modeling other facets of EBV biology. The two main shortcomings are: 1. the lack of human epithelial cells, which does not allow for the complete examination of EBV life cycle stages including viral titers during entry into the human body or during shedding into the saliva (Tsao et al., 2012), and 2. the limited differentiation from naïve B cells to memory and plasma B cells, which compromises both the humoral immune responses to EBV infection and the access of the virus to lower latency programs besides latency III (Thorley-Lawson and Allday, 2008). These aspects might be better modeled in monkeys, which harbor close relatives of EBV.

Along these lines, a lymphocryptovirus in rhesus macaques (rhLCV) has been explored. This virus infects through oral transmission, and causes acute and persistent infection, as well as tumorigenesis in immune suppressed monkeys (Moghaddam et al., 1997; Rivailler et al., 2004). This virus was entirely sequenced and seems collinearly homologous to EBV (Rivailler et al., 2002). rhLCV rhBARF1 was deleted to remove the colony stimulating factor-1 (CSF-1) blockade (Ohashi et al., 2012). The recombinant virus replicated less efficiently during acute and persistent infection in immune competent monkeys, but not in immune suppressed monkeys. However, the exact mechanism of CSF-1 triggered immune control of rhLCV was not identified. Nevertheless, cell-mediated and humoral immune responses against rhLCV have been reported in macaques; however, their protective value remains unexplored in this in vivo system (Fogg et al., 2005; Fogg et al., 2006; Orlova et al., 2011a; Orlova et al., 2011b). The difficulty in manipulating the macaque immune system to interrogate the functional relevance of rhLCV-specific immune responses points to the limitation of this in vivo system. Indeed, immune suppression for enhanced tumorigenesis and for the analysis of mutant rhLCVs was mainly achieved by co-infection with simian human immunodeficiency virus (SHIV) (Rivailler et al., 2004; Ohashi et al., 2012), which causes more alterations in the immune system than just immune suppression. Therefore, selective depletion of immune compartments is desirable to dissect the contribution of distinct cellular compartments to the immune control of lymphocryptoviruses.

9. Antibody depletion of EBV specific immune control

For this purpose, we and others have explored antibody depletion of immune compartments in HIS mice; as a small animal model, they are more amenable to these manipulations. The depleting dose (depending on the antibody avidity and isotype) and the reoccurrence time for the depleted immune compartment need to be established for each depleting antibody. For T cell depletion, we established a protocol where 50μg of an anti-CD8 antibody (OKT8; mIgG2a) and 100μg of an anti-CD4 (OKT4; mIgG2b or HP2/6; mIgG2a) are injected intraperitoneally per day on 3 consecutive days prior to EBV infection. Since T cell reconstitution reemerges 14 days after depletion, this depletion protocol needs to be repeated biweekly (Strowig et al., 2009). For the depletion of $CD4^+$ or $CD8^+$ T cells, these antibodies can also be used individually (Strowig et al., 2009; Chijioke et al., 2013). Billerbeck et al. depleted these subsets using 100μg of each antibody and repeating the depletion every 10 days (Billerbeck et al., 2013). Alternatively, Yajima and colleagues depleted T cells with an anti-CD3 antibody (OKT3; mIgG2a) by daily intravenous injections of 2 μg per mouse (Yajima et al., 2009). For $CD8^+$ T cell depletion they employed the anti-CD8 antibody B9.11 (mIgG1) using the same protocol. While these depleting antibodies all have mouse constant regions, $CD8^+$ T cells could also be eliminated by injecting the mouse-human hybrid antibody cM-T807 (huIgG1) 20 μg intravenously or 10 μg intraperitoneally every 3 days (Gorantla et al., 2010). In addition to T cells, NK cells have been also depleted in HIS mice. The NKp46 specific antibody BAB281 (mIgG1) was applied on 3 consecutive days with an intraperitoneal dose of 100 μg per mouse (Chijioke et al., 2013). Depletion was sustained for 4 weeks after this treatment. Finally, human DC populations have also been targeted by antibody depletion in HIS mice. Due to their fast reemergence after depletion, a reduction of $CD141^+$ conventional DCs was only achieved for 2 days (Meixlsperger et al., 2013). This transient depletion was induced by intraperitoneal injection of 10 μg of the anti-Clec9A antibody 8F9 (mIgG2a). Therefore, administering antibodies to HIS mice can transiently deplete reconstituted human immune system components, and the protective value of these compartments in immune responses against EBV can be elucidated further. However, more sophisticated manipulations of the human immune system components in HIS mice need to be attempted to probe immune control of EBV in more detail.

10. Conclusions and outlook

HIS mice provide a novel and interesting model to examine immune cell interactions in vivo. Multiple avenues for the improvement of this mouse model system, however, remain (Figure 1). The engraftment and maintenance of the human compartment could be improved by the genetic introduction of key human chemokines and cytokines that promote different cell types, as has been demonstrated for myeloid cell differentiation where the knock-in expression of human TPO, GM-CSF, and IL-3 resulted in improved myeloid populations (Willinger et al., 2011; Rongvaux et al., 2013). Animals expressing multiple human HLA transgenes would provide more complete thymic T cell education and likely result in improved specific immune responses, as has been demonstrated in the HLA-A2 transgenic huNSG animals infected with EBV (Strowig et al., 2009). Additionally, genetic manipulation of the reconstituting human immune system components prior to EBV infection should be examined. Viral transduction of the $CD34^{+}$ hematopoietic progenitor cells should allow the overexpression or down-modulation of distinct receptors or transcription factors in subsequently reconstituting human immune system components. This could provide the HIS mouse counterpart of traditional knockout and transgenic mice and dissect the distinct molecular requirements for protective immune compartments, including DCs, NK cells, and T cells during EBV infection.

Combined with the established infection models with mutant EBV viruses and antibody-mediated immune compartment depletion, such studies on the molecular basis for EBV immune control should allow us to narrow down individuals at risk for developing EBV-associated diseases who could benefit from vaccination. Similar to the human papilloma virus vaccine, EBV negative adolescents with a significant risk for developing IM after primary infection might be an ideal patient population. Given the seriousness of EBV-associated diseases, HIS mice will be of importance in developing the right vaccine strategies for patients with unmet medical needs.

Acknowledgements

Our research is supported by the Cancer Research Switzerland (KFS-3234-08-2013), the Association for International Cancer Research (11-0516), KFSP^{MS} and KFSP^{HLD} of the University of Zurich, the Baugarten Foundation, the Sobek Foundation, Fondation Acteria, and the Swiss National Science Foundation (310030_143979 and CRSII3_136241).

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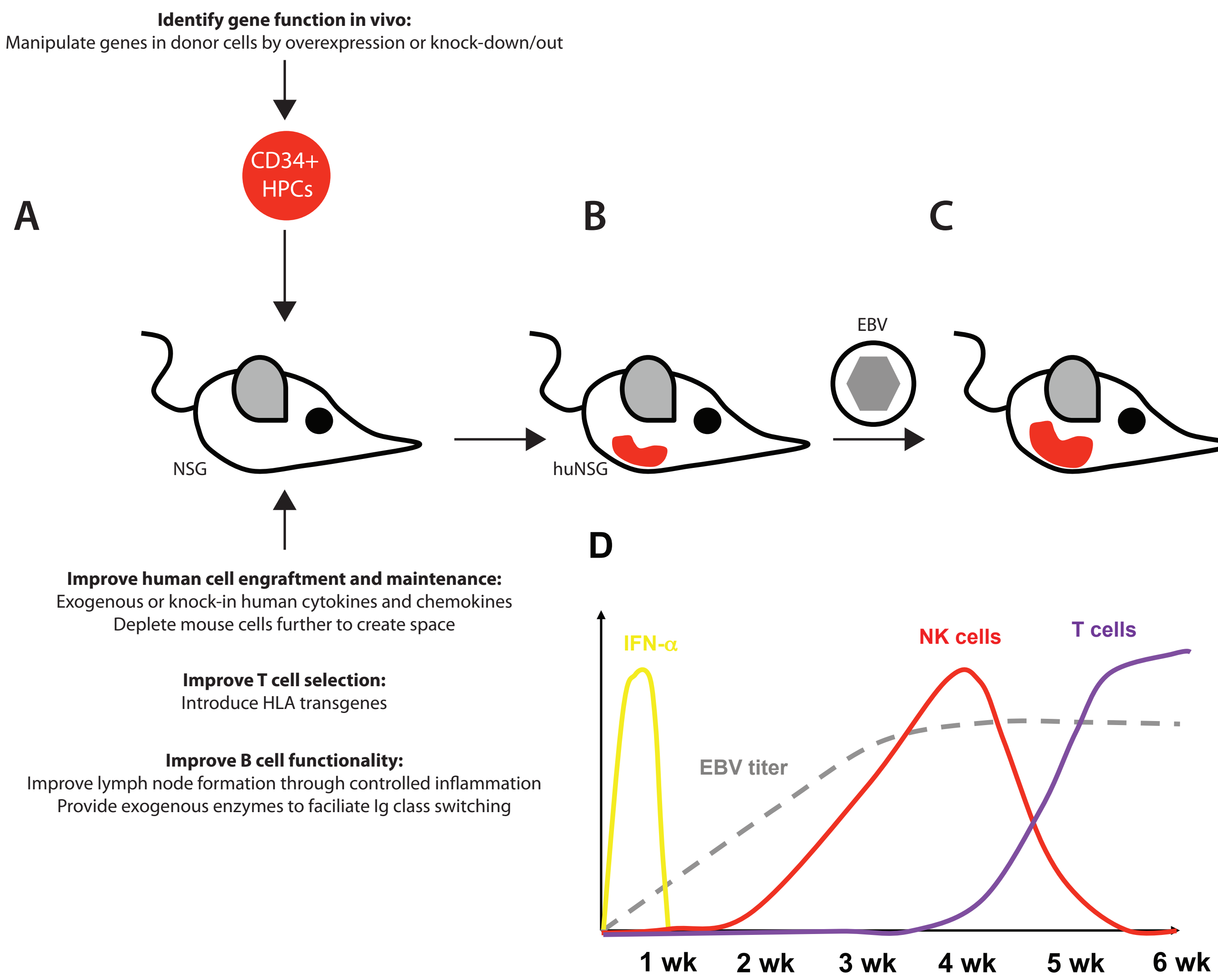
Figure legend

Figure 1: HIS mice recapitulate aspects of EBV specific immune control. Neonatal transfer of $CD34^+$ hematopoietic progenitor cells ($CD34^+$ HPCs) into immune compromised mice, i.e. NOD-*scid* $\gamma_c^{-/-}$ mice (NSG), allows for the reconstitution of human immune system components within three months **(A).** Some improvements that have or will be applied in the near future are listed. These HIS mice, i.e. huNSG mice, can then be infected with EBV **(B)**, leading to splenomegaly **(C)**. Following infection, early protective IFN-α responses by pDCs, NK cell responses to lytic EBV infection (peaking at four weeks after infection), and protective T cell expansion can be observed **(D).**


Identify gene function in vivo:
Manipulate genes in donor cells by overexpression or knock-down/out
CD34+ HPCs
A
B
C
EBV
NSG
huNSG
Improve human cell engraftment and maintenance:
Exogenous or knock-in human cytokines and chemokines
Deplete mouse cells further to create space
Improve T cell selection:
Introduce HLA transgenes
Improve B cell functionality:
Improve lymph node formation through controlled inflammation
Provide exogenous enzymes to faciliate Ig class switching
D
IFN-α
NK cells
T cells
EBV titer
1 wk
2 wk
3 wk
4 wk
5 wk
6 wk